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Uric Acid Accumulation in the Solitary Ascidian *Corella inflata*

CHARLES C. LAMBERT,[1,2*] GRETCHEN LAMBERT,[1,2]
GUY CRUNDWELL,[3] AND KATHERINE KANTARDJIEFF[3]
[1]*Department of Biological Science, California State University Fullerton, Fullerton, California 92834-6850*
[2]*Friday Harbor Laboratories, Friday Harbor, Washington 98250*
[3]*W.M. Keck Center for Molecular Structure and Department of Chemistry/Biochemistry, California State University Fullerton, Fullerton, California 92834-6866*

ABSTRACT Like many other ascidians, *Corella inflata* sequesters uric acid. We have identified microcrystals of uric acid by X-ray powder diffraction, by the characteristic UV absorption for urates at 292 nm which is abolished by uricase and by methenamine-silver staining in situ. The uric acid is precipitated in birefringent spherulites which are formed intracellularly within vacuoles of nephrocyte cells. Each spherulite is composed of hundreds of thin crystals radially arranged. These 12-μm spherulites accumulate in white structures clearly visible with the naked eye for which we suggest the designation *tophus*, a medical term used to describe any amorphous concretion. In adults, these tophi are found in several tissues including the body wall, gonads, digestive tract and branchial sac trabeculae. The tophi are often over 200 μm in diameter and include spherulites within nephrocytes as well as free spherulites. Each tophus is surrounded by an epithelium enclosing a thick layer of fibrous extracellular matrix. The first birefringent spherulites are detected 3 days after fertilization, a day before the branchial siphon opens and feeding begins. They form in vacuoles of nephrocytes which do not circulate with the blood but appear to be in fixed locations in the organism. Once begun, the quantity of stored urate increases throughout life which is typically less than a year. *J. Exp. Zool. 282:323–331, 1998.*

It has long been known that a number of ascidians sequester urates or other purines in their bodies over long periods of time (Goodbody, '65). This is in addition to excreting nitrogenous waste in the form of ammonia and urea (Goodbody, '57; Markus and Lambert, '83). Purine storage has been considered an evolutionary accident involving the loss of enzymes responsible for purine degradation (Goodbody, '65). Others have suggested that the purines are instead stored for use in nucleic acid synthesis or other uses (Nolfi, '70) or even as structural elements (Lambert et al., '90).

In the Molgulidae urates are stored in the single large multicellular renal sac which also contains calcium oxalate in the form of weddellite (Saffo and Lowenstam, '78). The renal sac has no openings but the amount of stored crystalline material changes with time, suggesting utilization along with precipitation (Nolfi, '70; Saffo, '88). Symbiotic, fungus-like organisms play a role in these changes (Saffo, '82, '90). Most members of the family Ascidiidae have numerous renal vesicles covering the entire left side of the body in association with the gut. These multicellular vesicles have spacious cavities containing concretions which are often, but not always, of uric acid (Goodbody, '65). Several members of the genus *Corella* have scattered white inclusions in the body wall which are larger than the renal vesicles and have been considered to be urates (Markus and Lambert, '83), but no information on their chemical composition has been available. In this report we demonstrate that these white inclusions in *Corella* indeed contain uric acid in the form of crystals organized into spheroids (spherulites) contained in netlike structures of fibrous connective tissue. The spheroids begin to form intracellularly within nephrocytes shortly after metamorphosis. The number of nephrocytes with crystalline inclusions increases rapidly. Once begun, the quantity of uric acid increases throughout the lifespan of the animal, which is less than a year (Lambert, '68).

*Correspondence to: Charles C. Lambert, Dept. of Biol. Sci., California State Univ. Fullerton, Fullerton, CA 92834-6850. E-mail: clambert@fullerton.edu
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MATERIALS AND METHODS

Corella inflata (order Phebobranchia, family Corellidae; see Lambert et al., '81 for clarification of systematics) was collected from floating docks in Friday Harbor, Washington and held in running sea water until used. Gametes for culture were obtained by light-induced spawning (Lambert et al., '81). Fertilized eggs were cultured in glass dishes on the sea table at 12–14°C. Metamorphosis occurred on the sides and bottom of the dish as well as on the surface film. The surface-metamorphosed individuals were removed when changing the sea water after 3 days. Individuals were dissected from the dishes and placed on a slide under a supported coverslip and viewed and photographed under differential interference contrast and polarization optics in an Olympus BHS microscope.

Portions of body wall were fixed in 2% glutaraldehyde in 0.4 M phosphate buffer pH 7 containing 12% sucrose for 1 hr at room temperature. Following this the tissue was rinsed in buffer and post-fixed for 1 hr in 1% OsO_4 in the same sucrose-supplemented buffer as before. The tissue was then dehydrated in an ethanol series, passed through propylene oxide and embedded in Epon resin for sectioning.

Histochemical determination of urate in the tophi utilized the methenamine silver technique (Bancroft, '75) with dissected portions of body wall.

Absorption spectra were obtained from samples homogenized in 0.7 M glycine buffer pH 9.4 followed by centrifugation at 5,000*g* for 5 min to remove particulates. Absorbance was read in a Beckman DU spectrophotometer equipped with a UV light source. Uricase (Sigma, St. Louis, MO) was added and the absorption spectrum repeated. The quantity of urate was determined by cutting open animals, blotting away as much water as possible, weighing the animals, homogenizing in glycine buffer and determining the absorbancy at 292 nm followed by uricase digestion and a second absorbance reading as outlined in Sigma procedure 292-UV.

Ammonia production was determined by incubating clean specimens of *Corella* in millipore filtered sea water for 24 hr followed by determination of ammonia using indophenol blue production from ammonia with commercially prepared reagents from Boehringer Mannheim (Indianapolis, IN).

X-ray powder diffraction was performed by packing samples of dissected tophi into 0.5-mm glass capillaries. The capillaries were sealed with paraffin wax, then mounted on the goniometer of a Siemens three-circle diffractometer. X-rays were generated with Mo Kα radiation (λ = 0.710746 Å) and were collected on a charged coupled device (CCD) image detector 4.97 cm away from the sample. For data collection, a series of 36 CCD images was collected for each sample by rotating the capillary 10 degrees per image around its primary axis. After the data runs were completed, the images were combined and subtracted against a background spectrum that consisted of an identical data collection run on an empty capillary. The first sample investigated was 0.4 g of commercially pure uric acid (Sigma). The second sample consisted of dissected tophi-rich portions from *Corella inflata* stored in ethanol.

RESULTS

Structure of tophi and spherulites

The presence of the abundant macroscopic white structures, hereafter termed tophi, are easily visible with the unaided eye through the transparent tunic (Fig. 1). These variously shaped structures up to 0.2 mm in diameter are scattered throughout the body wall, on the surface of the gut, embedded in the testis and even in the trabeculae supporting the branchial sac. In sections, it appears that the tophi are often associated with blood vessels. Low-power magnification of these bodies (Fig. 2) reveal that they are composed of numerous spherical structures similar in appearance to the 10–20 μm spherulites identified in certain types of gout in humans (Fiechtner and Simkin, '81). The maximum diameter of the *Corella* spherulite is 12 μm. Under polarization optics each spherulite is seen to be composed of numerous radially arranged birefringent crystals (Fig. 3).

Identification of urate in tophi

The birefringent crystals originally suggested that calcium carbonate might be precipitated as in other ascidians (Lambert et al., '90). However, the addition of 4N H_2SO_4 to the concretions failed to release the diagnostic bubbles of CO_2. Therefore we undertook tests to investigate the presence of urates, which form concretions in some other ascidians (Goodbody, '65, '74). The concretions slowly dissolve in sea water but are insoluble in 4N H_2SO_4 and 95% ethanol. They readily dissolve in pH 9.4 glycine buffer. The absorption spectrum of a glycine buffer extract of body wall shows the characteristic absorption

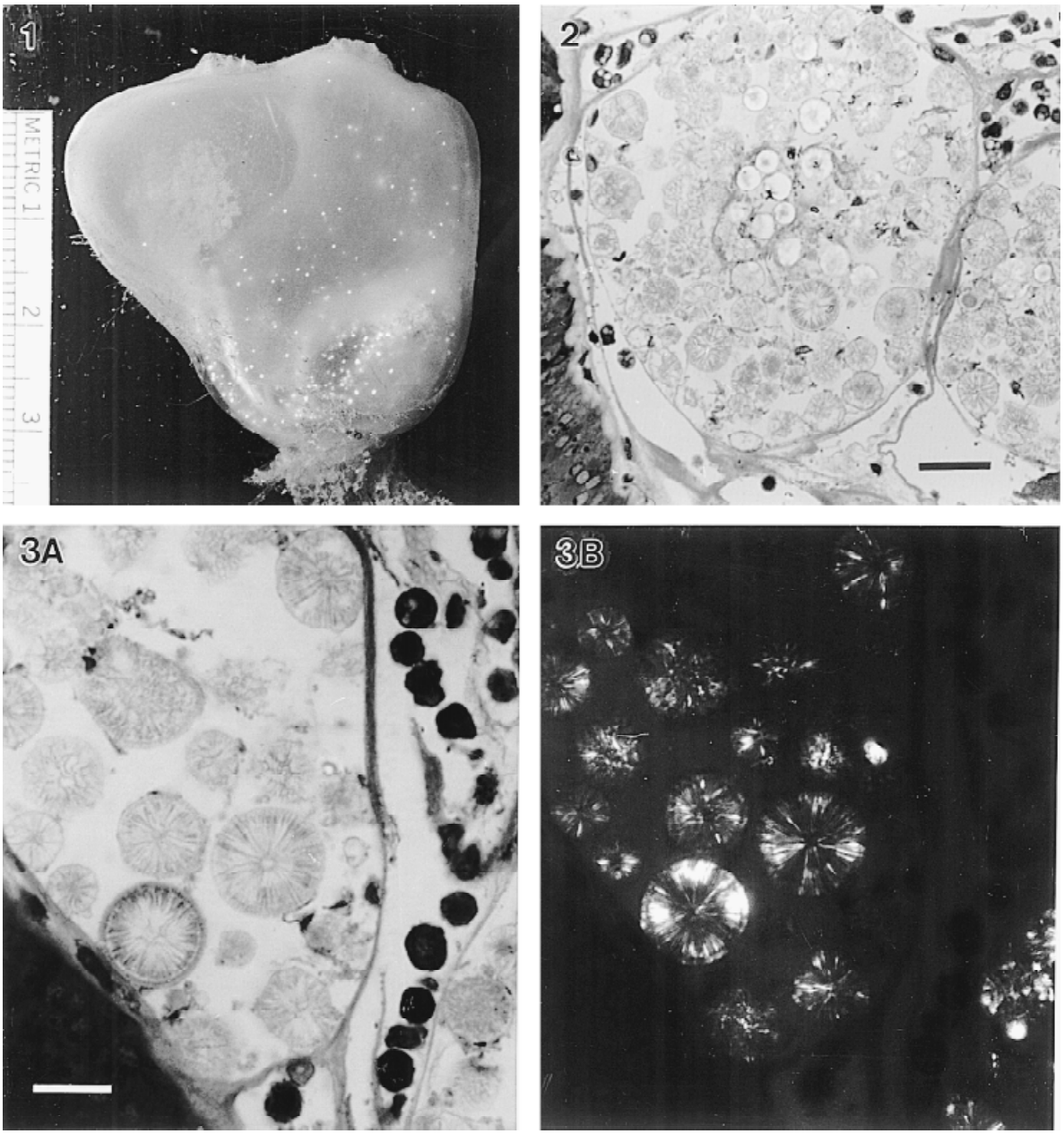

Fig. 1. *Corella inflata* whole living animal. The small white spots are tophi in the body wall and other locations.

Fig. 2. A 1 μm section of a tophus in the body wall. Bar = 20 μm.

Fig. 3. *C. inflata* tophus. Epon embedded body wall was sectioned at 1 μm and viewed with bright field (**A**) or polarizing (**B**) optics. Bar = 20 μm.

peak at 292 nm, which is diagnostic of urates. In addition, the peak is abolished by incubation with commercial uricase, confirming this identification (Fig. 4). The methenamine silver histochemical test showed the presence of urate in the aggregate components of the tophus (Fig. 5) and gave an additional confirmation of the presence of urate in these structures. X-ray powder diffraction spectra of commercial uric acid and *Corella inflata* tophi show similar 2θ Bragg reflections (Fig. 6). The major peak at d = 3.13 Å is clearly visible in both samples as are peaks at 3.8 Å, 5.7 Å, and 6.4 Å. These peaks correspond with the published literature values for uric acid (Shirley and Sutor, '68). The sample of *Corella inflata* shows considerable amounts of background noise starting from 3.7 Å and higher due to amorphous X-ray scattering. This was not surprising since the sample

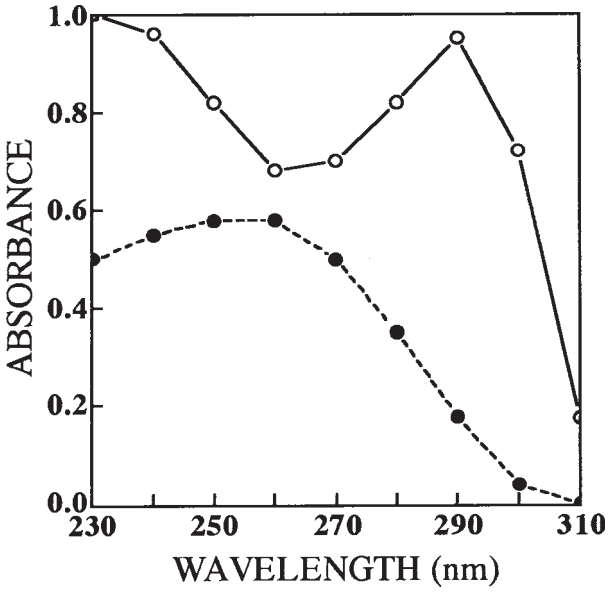

Fig. 4. Absorption spectrum of *Corella inflata* whole body extract. Mature adults were removed from their tunics, homogenized in pH 9.5 glycine buffer, clarified by centrifugation and the absorbance determined with (solid circles) and without (open circles) uricase digestion.

consisted primarily of cellular and organic material with only minute quantities of microcrystalline uric acid.

Using a red plate compensator, we determined that these crystals are positively birefringent; they are blue parallel to the plane of the compensator and yellow perpendicular to this plane. However, pure salts of uric acid are negatively birefringent and appear yellow parallel to the plane of first-order red plate compensator (Fiechtner and Simkin, '81). We synthesized crystals of the sodium and potassium salts of uric acid (Fiechtner and Simkin, '81) and found that they were negatively birefringent as expected. It may be that protein incorporated with the crystals changes the sign of polarization. In order to test the effect of protein on this property, sodium urate was allowed to precipitate in the presence of gelatin. Many of these crystals were positively birefringent.

Nephrocytes

Examination of the spherulites with differential interference optics disclosed that they are contained intracellularly within large cells up to 24 μm in diameter (Fig. 7). Such vacuolated cells with concretions are called nephrocytes (George, '39). Each nephrocyte initially contains a single spherulite; later there are often 3 or 4 in a much larger cell. Large nephrocytes are often lumpy in

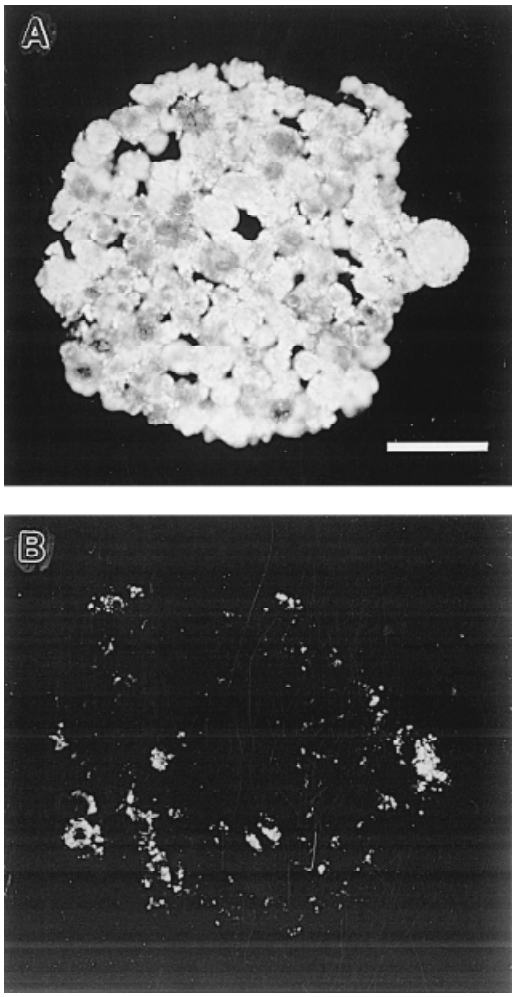

Fig. 5. Methenamine silver stained tophus from *C. inflata* body wall. Tophus whole mount viewed by reflected light. (**A**) control tophus unstained; (**B**) tophus stained with methenamine silver; note that all the spherulites have been blackened, indicating the presence of urate. Bar = 10 μm.

outline (Fig. 7) because of their massive inclusions, each apparently carried in the center of a large vacuole. The nephrocytes do not circulate with the blood but appear to remain in fixed positions within the body of newly metamorphosed individuals.

Nephrocytes with multiple spherulites are also found in adult *Corella* tophi but not all of the spherulites are intracellular. Many of the largest appear to be extracellular. Within smaller adults the proportion of intracellular spherulites is higher than in larger, presumably older, individuals. Occasionally, spherulite-containing small nephrocytes were obtained by cardiac puncture, but large nephrocytes with multiple spherulites were never obtained from the circulating blood.

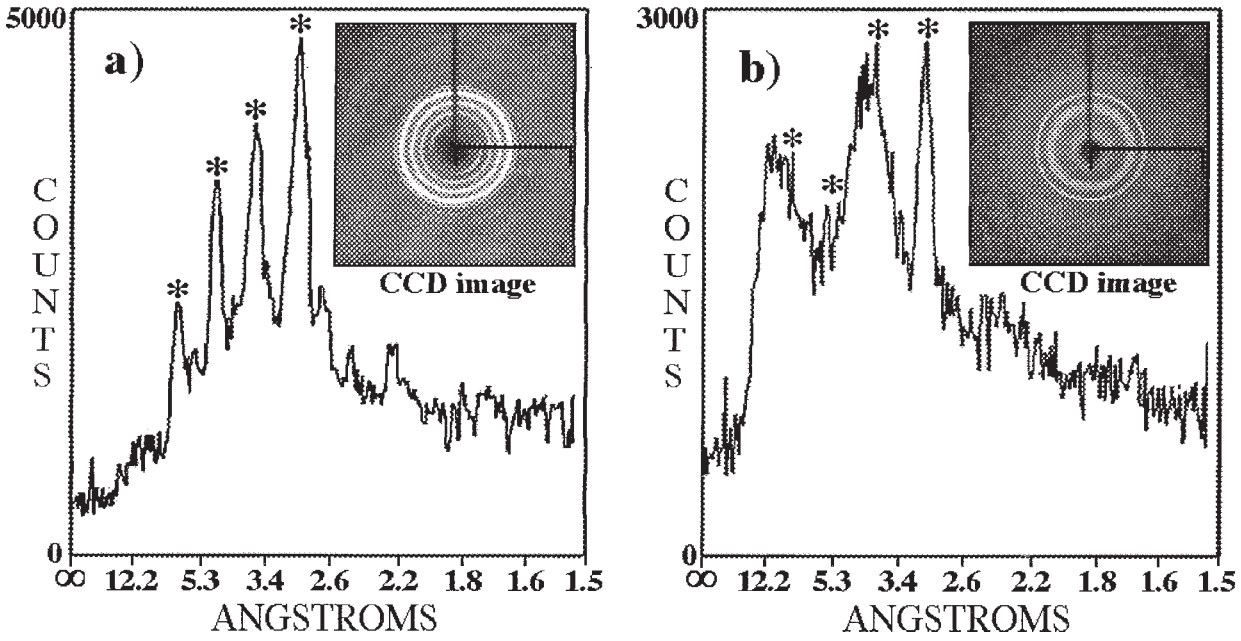

Fig. 6. X-ray powder diffraction spectra from collected CCD images (inserts) of commercial uric acid (left) and whole *Corella inflata* tophi (right). The asterisks represent the most visible observed reflections.

Ultrastructure of spherulites

Each spherulite apparently aggregates around a singular nucleation site. In Fig. 8 the crystals are missing but a fibrous radially organized matrix outlines the former positions of the crystals and also the periphery of the spherulite. Since the crystals were present in the thick sections depicted in Figs. 2 and 3, fixation, dehydration and embedding did not remove them. Possibly the crystals were lost during thin sectioning (Bagnara, '66) or were sublimed by the heat of the electron beam (R. Cloney, University of Washington, pers. comm.), as uric acid is decomposed by heat without melting (Windholz, '76). Each tophus (group of spherulites) is bounded by an epithelium enclosing a fibrous extracellular matrix that can be quite thick (Figs. 9, 10).

Kinetics of urate accumulation

Naturally spawned eggs of *C. inflata* (Lambert et al., '81) were cultured in the laboratory at 12°C, the normal sea temperature. The tadpoles hatch about 24 hr after fertilization. Metamorphosis occurs during the second day (Lambert et al., '95), following which the sub-adult undergoes extensive reorganization before the oral siphon opens and feeding begins on the fourth day. Embryos were cultured in glass dishes and examined daily with a dissection microscope followed by careful removal of a few specimens for examination under polarizing optics in the compound microscope. On the third day after fertilization, the first birefringent crystals were detected. By the next day when feeding began there were 8–12 distinct birefringent loci, some with multiple spherulites (Fig. 11). The first crystals generally appeared below the stomach in the heart region; successive crystals appeared throughout the body. Spherulites were added at a regular rate in the laboratory during the first weeks of development.

The time course of urate accumulation of postmetamorphic individuals was also followed in field-collected animals. They were carefully blotted, weighed, and the amount of urate determined as a function of body size. Even the smallest field-collected animals have significant urate levels. The quantity of urate increases regularly throughout the life of the animals as shown by the linear regression of urate content and wet weight (Fig. 12).

Allopurinol and nephrocyte formation

In humans, gouty arthritis is a painful disease characterized by the deposition of uric acid in joints (Fiechtner and Simkin, '81). Allopurinol, a xanthine oxidase inhibitor, is one of the most prescribed drugs that results in a reduction in uric acid formation (Ralstom, '93). Since we hypoth-

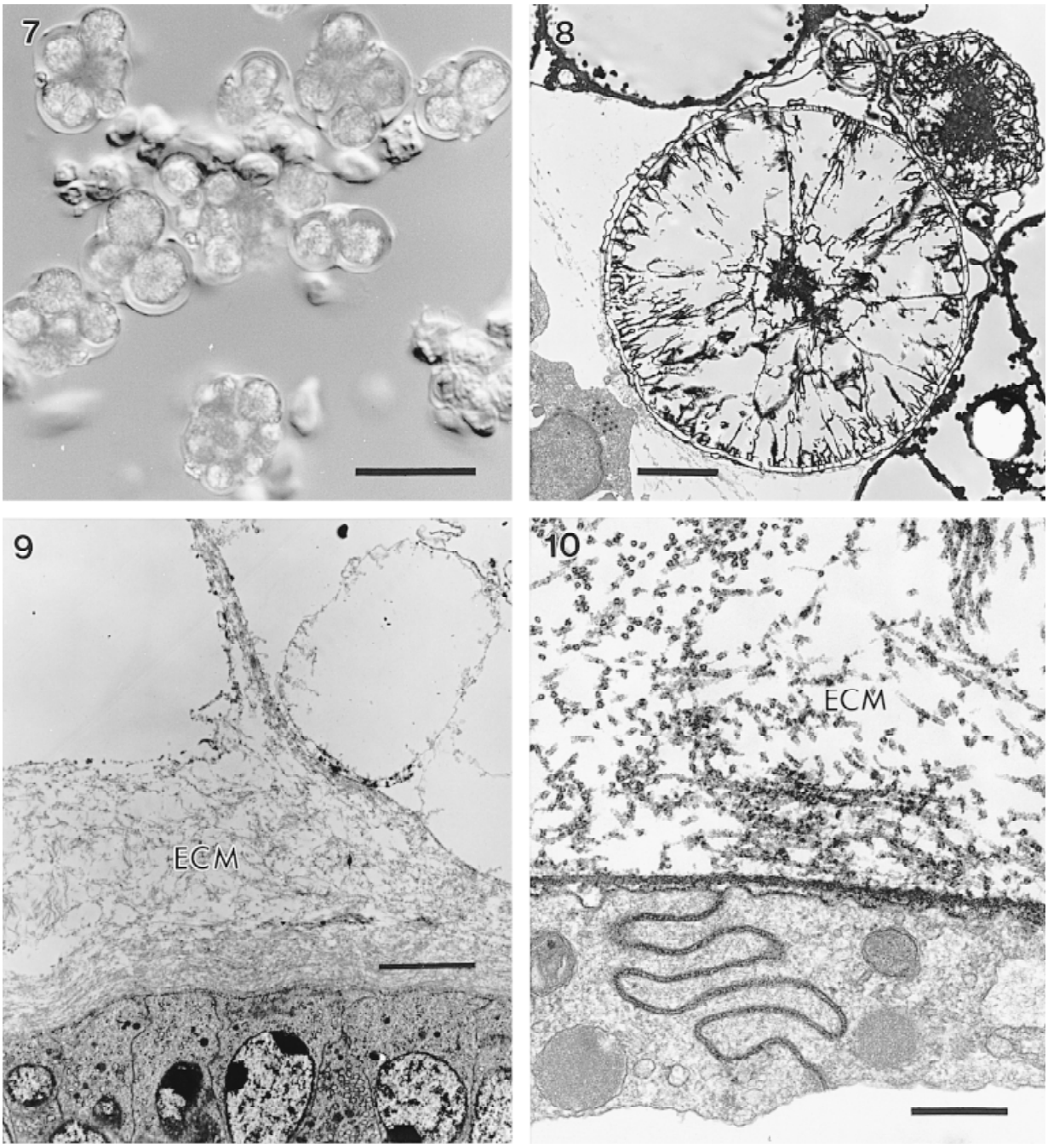

Fig. 7. Nephrocytes of living *C. inflata* containing several spherulites. Differential interference contrast (DIC) image. Bar = 20 μm.

Fig. 8. Electron microscope image of spherulite organic support structure. The urate crystals evident in Figs. 2 and 3 were either lost in thin sectioning or sublimed in the electron beam, leaving a radially organized supporting structure. Bar = 2 μm.

Figs. 9 and 10. Electron microscope images of portions of tophus periphery. Note well-preserved epithelium and thick layer of extracellular matrix (ECM). Fig. 9: bar = 4 μm; Fig. 10: bar = 0.5 μm.

esize that the spherulites of *Corella* are primarily urate crystals, we thought it worthwhile to examine the effect of this drug on urate production in the ascidian. Accordingly we incubated newly metamorphosed *Corella* embryos in 1 or 3 mM allopurinol in sea water and found that at the higher concentration it suppressed the formation of birefringent concretions, but also

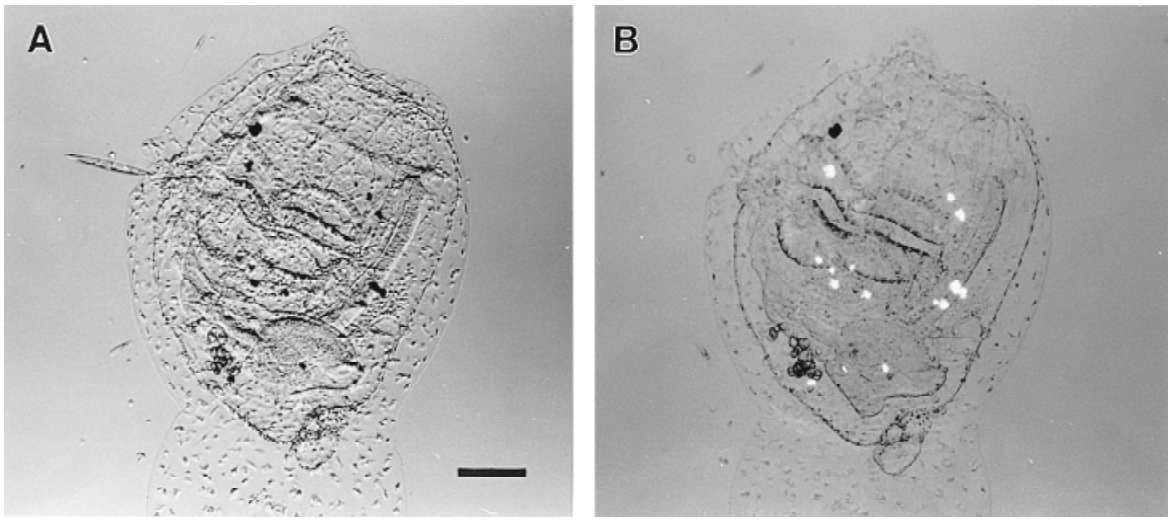

Fig. 11. Whole, living *C. inflata* sub-adult four days after fertilization. (**A**) DIC image. (**B**) Polarization image. Note bright birefringent spherulites. Bar = 100 μm.

greatly diminished the normal growth as compared to control embryos. The drug allowed spherulite formation at 1 mM and had no obvious effect upon growth.

Nitrogen excretion

Since not all ascidians sequester urates but do excrete other forms of nitrogen in the form of ammonia and urea (Markus and Lambert, '83), and *Corella* in addition uses ammonia for buoyancy of the follicle cells surrounding the eggs (Lambert and Lambert, '78), we also measured ammonia excretion rates to determine whether this was a component of nitrogen excretion. *Corella inflata* releases ammonia into the sea water at the rate of 15 μg/hr/g dry weight. This is less than *Ciona intestinalis*, which excretes 86 μg/hr/g dry weight (Markus and Lambert, '83). Nevertheless, ammonia remains an important end product of protein catabolism in *Corella inflata.* The fact that some ammonia is used in egg flotation and in addition *Corella* stores uric acid probably accounts for the diminished ammonia excretion as compared to *Ciona*, which neither stores uric acid (Goodbody, '57) nor produces buoyant eggs.

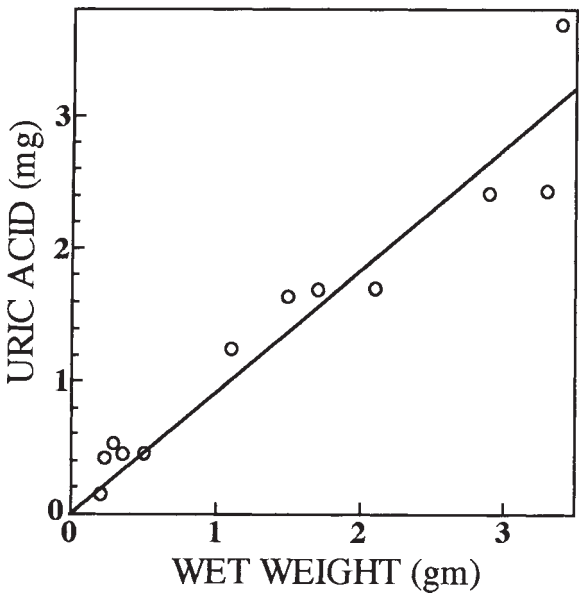

Fig. 12. Kinetics of urate accumulation in *C. inflata.* Animals were prepared as in the Methods section and the wet weight and urate content determined from field collected adults.

DISCUSSION

The characteristic absorption spectrum with a peak at 292 nm, coupled with the ability to abolish the peak with uricase, clearly proves the presence of large quantities of urate in *C. inflata*. Methenamine staining of the spherulites for urate supports these as the storage site. Definitive identification of the needle-like crystals within the spherulites as uric acid was made by X-ray powder diffraction. Interestingly, the crystals are positively, rather than negatively, birefringent. Authentic uric acid is negatively birefringent, and we synthesized the sodium and potassium salts of uric acid to confirm their negative birefringence. Probably the urate in *Corella* precipitates in the presence of protein within the nephrocyte vacu-

ole. When we precipitated the sodium salt of uric acid in the presence of gelatin we found many crystals with positive birefringence. It is also possible that the uric acid is in the monohydrate form as mentioned in Shirley and Sutor ('68). The power diffraction patterns for the anhydrous and hydrated forms of uric acid are similar.

Allopurinol inhibits formation of the spherulites at 3 mM but at 1 mM had no obvious effect. Since allopurinol is used specifically to prevent uric acid precipitation in humans (Ralstom, '93), this further supports the identification of the spherulites as uric acid. However, since the drug also slowed growth, the effect may not be specific in ascidians. It is possible that some concentration of the drug lower than 3 mM but higher than 1 mM might block deposition of the spherulites without inhibiting growth. As expected, ammonia production by *Corella* was less than half the value in *Ciona*, which neither sequesters urates (Goodbody, '65) nor uses ammonia for egg flotation (Lambert and Lambert, '78).

The spherulites are first formed in nephrocytes even before feeding begins. Although we have found these cells in the heart, for the most part the cells do not circulate with the blood but are in fixed locations in the body. Such fixed collections of nephrocytes are also found in *Ecteinascidia conklini*, *Clavelina oblonga*, and *Botrylloides niger*, where they may form white flecks (George, '36).

Previously, urate storage in ascidians was known to occur in two major locations: the single large renal sac in molgulids, and the multiple renal vesicles found in *Herdmania* and ascidiids. Renal vesicles are composed of a multicellular epithelium surrounding a central vacuole in which the concretion is formed (George, '36; Goodbody, '65). They accumulate around the gut of the adult. The vesicles always have concretions but these may or may not contain urates (Goodbody, '65). For instance, both *Ascidia paratropa* and *Ascidia callosa* have vesicles with concretions but only *Ascidia paratropa* has the material with an absorption peak of 292 nm (C. Lambert, unpublished). The tophi of *Corella* are quite different from renal vesicles. They are much larger, surrounded by a thick extracellular matrix in addition to an epithelial layer and are scattered within the body wall, trabeculae and even in the ovary in contrast to the renal vesicles which are confined to the region of the gut. Thus the tophi are quite distinct structures which appear to be unique to certain *Corella* species.

The function of purine storage in ascidians has been a matter of considerable speculation. Goodbody ('65) considered storage as a form of excretion, while others have suggested that the stored urate might be liberated during periods of high purine demand (Nolfi, '70; Saffo, '88). An alternative explanation for the function of the white bodies might be structural (Lambert et al., '90). Calcium carbonate crystalline spicules help support the tunic and body wall of a number of solitary ascidians mostly from the family Pyuridae (Lambert et al., '90; Lambert, '92; Lambert and Lambert, '97). Spicules of calcium carbonate are also embedded in the tunic of many compound ascidians from the families Didemnidae and Polycitoridae (Lambert et al., '90). The tophi of *Corella* would certainly lend rigidity to the trabeculae which support the branchial sac. In addition, they could lend support to the body wall and tunic (Koehl, '82) in much the same way as the spicules of pyurids. *Corella inflata* has a very thin tunic as compared with most other phlebobranch ascidians; the tophi may provide structural support in this ascidian.

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